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Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function

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Predicted future CO_2 levels have been found to alter sensory responses and behaviour of marine fishes. Changes include increased boldness and activity, loss of behavioural lateralization, altered auditory preferences and impaired olfactory function[1–5]. Impaired olfactory function makes larval fish attracted to odours they normally avoid, including ones from predators and unfavourable habitats[1,3]. These behavioural alterations have significant effects on mortality that may have far-reaching implications for population replenishment, community structure and ecosystem function[2,6]. However, the underlying mechanism linking high CO_2 to these diverse responses has been unknown. Here we show that abnormal olfactory preferences and loss of behavioural lateralization exhibited by two species of larval coral reef fish exposed to high CO_2 can be rapidly and effectively reversed by treatment with an antagonist of the GABA-A receptor. GABA-A is a major neurotransmitter receptor in the vertebrate brain. Thus, our results indicate that high CO_2 interferes with neurotransmitter function, a hitherto unrecognized threat to marine populations and ecosystems. Given the ubiquity and conserved function of GABA-A receptors, we predict that rising CO_2 levels could cause sensory and behavioural impairment in a wide range of marine species, especially those that tightly control their acid–base balance through regulatory changes in HCO_3^- and Cl^- levels.

Recent studies have shown that larval fish develop behavioural and sensory abnormalities when exposed to CO_2 levels predicted to occur in the sea by the end of this century (700–900 μatm; refs 7,8). In light of the striking alterations in a wide range of behaviours, including reversal or loss of olfactory and auditory preferences, we proposed that inhibitory GABAergic transmission through GABA-A receptors may become excitatory after high-CO_2 exposure (Fig. 1). The GABA-A receptor, the main inhibitory neurotransmitter receptor in the vertebrate brain, has high conductivity for Cl^- and to a lesser extent for HCO_3^- (ref. 9). The normal situation in vertebrates is that ion gradients over the neuronal membrane are such that an opening of the GABA-A receptor causes an inflow of Cl^-, leading to hyperpolarization and inhibition of the neuron. However, in the fetal mammalian brain, and in some conditions of neuronal overactivity, such as in epilepsy, these ion gradients are reversed owing to an accumulation of Cl^- or HCO_3^- intracellularly. The result is that GABA-A receptor activation leads to depolarization and excitation of the neuron[10–12]. A key point is that the neuronal ion gradients for Cl^- are relatively close to electrochemical equilibrium[10], which is in contrast to the extreme transmembrane gradient for Na^+ and Ca^{2+} that drives most excitatory events in the brain. When exposed to high CO_2, marine fish regulate their acid–base balance to avoid acidosis by accumulating HCO_3^-, with compensatory reductions in Cl^- (refs 13,14). We propose that these regulatory changes in HCO_3^- and Cl^- levels during high-CO_2 exposure render some GABA-A receptors excitatory, thereby affecting behaviour and causing dramatic shifts in sensory preferences.

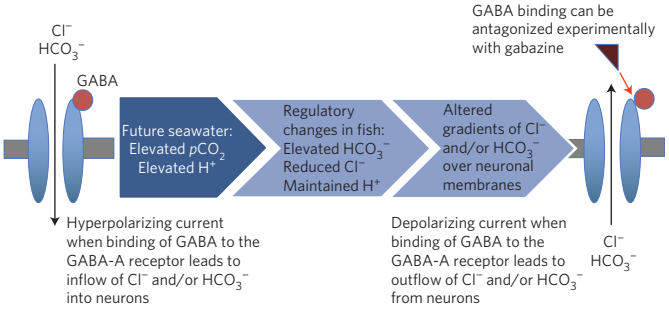

Figure 1 | Proposed response of GABA-A receptor function to elevated sea water pCO_2. This GABA-gated ion channel has conductance for Cl^- and HCO_3^-. Normally, ion gradients are such that channel opening results in Cl^- and HCO_3^- inflow, causing membrane hyperpolarization and inhibited neural activity. Elevated sea water pCO_2 leads to ion-regulatory adjustments in blood and tissues that affect transmembrane gradients for Cl^- and/or HCO_3^- in some neurons. GABA-A receptors thereby become depolarizing and excitatory, resulting in behavioural abnormalities. We here provide support for this hypothesis by showing that the behavioural effects of high CO_2 are reversed by treatment with a GABA-A receptor antagonist (gabazine).

To test the involvement of GABA-A receptor function in the sensory and behavioural changes shown by fish exposed to high CO_2, we treated settlement-stage larvae with gabazine, a specific GABA-A receptor antagonist[15]. Clownfish (*Amphiprion percula*) larvae were reared for 11 days from hatching until the end of their larval phase in either control or high CO_2 environments (~450 and ~900 μatm CO_2, respectively), and then tested for their olfactory responses to predator odour. Settlement-stage damselfish (*Neopomacentrus azysron*) caught in the wild were exposed to the

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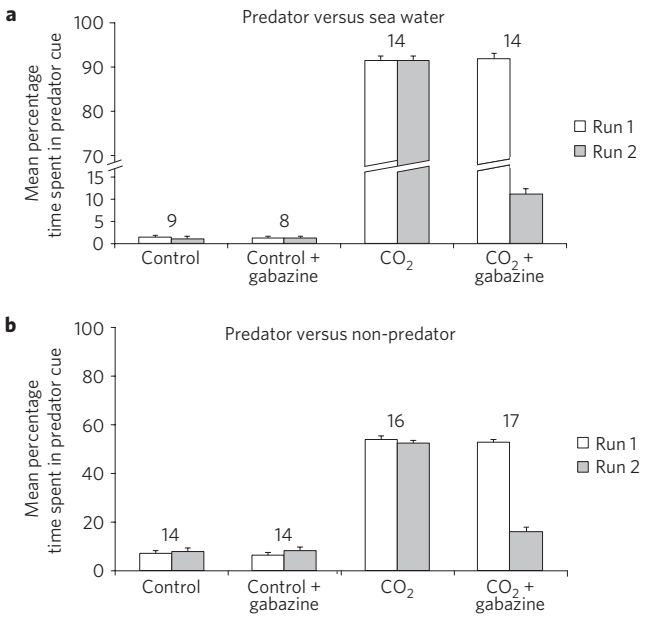

Figure 2 | Olfactory ability of larval clownfish (*Amphiprion percula*) is impaired by high CO_2 and restored by a GABA-A receptor antagonist. **a**,**b**, In a two-channel flume chamber, fish were given the choice of swimming in sea water with or without predator odour (**a**), or in sea water with predator odour versus sea water with non-predator odour (**b**). Olfactory preferences of larvae reared in control (~450 μatm) or high CO_2 (~900 μatm) were tested twice: before (run 1) or after (run 2) a holding period (30 min) in control sea water or sea water containing gabazine. Values are means ±s.e.m. The numbers of fish are indicated above the columns.

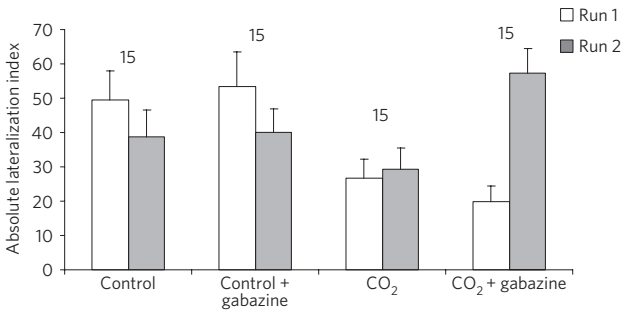

Figure 3 | Behavioural lateralization of larval damselfish (*Neopomacentrus azysron*) is impaired by high CO_2 and restored by a GABA-A receptor antagonist. Fish were required to make ten choices of turning left or right in a T-shaped maze. Absolute lateralization ranges from 0 to 100: zero indicates an equal number of left and right turns whereas 100 indicates all turns in one direction. Larvae exposed to control (~450 μatm) or high CO_2 (~900 μatm) were tested twice: before (run 1) or after (run 2) a holding period (30 min) in control sea water or sea water containing gabazine. $N = 15$ for each treatment. Values are means ±s.e.m.

same CO_2 treatments for four days and then tested for lateralization of preferred direction of turning, which is a direct test of brain function. In both cases the involvement of altered GABA-A receptor function was tested by treating half the fish in sea water containing gabazine for 30 min immediately before the behavioural tests.

Clownfish larvae reared in high CO_2 exhibited a dramatic shift in olfactory preference, which was almost completely corrected by gabazine treatment (Fig. 2). In the first experiment, where larvae were given the choice of a water stream containing the odour of a common predator, the rockcod *Cephalopholis cyanostigma*, versus a water stream containing no additional odour, clownfish larvae reared in control water were strongly repelled from the predator odour, and this response was not affected by gabazine treatment (Fig. 2a). In contrast, clownfish larvae reared at ~900 μatm CO_2 were strongly attracted to the predator odour (>90% of time), as has been observed in previous experiments[2,3]. However, this response was reversed following gabazine treatment, with larvae spending less than 12% of their time in the water stream containing the predator odour (Fig. 2a). In the second experiment, where clownfish larvae were given the choice of a water stream containing the odour of the predatory rockcod versus a water stream containing the odour of a herbivorous fish, *Siganus corallinus*, the larvae reared in control water distinguished between the odours and were strongly repelled from the predator odour (Fig. 2b). As with the first experiment, this response was not affected by gabazine treatment. In contrast, clownfish larvae reared at ~900 μatm CO_2 could not distinguish between the odours and spent approximately equal times in the predator and non-predator odours (Fig. 2b). This response was reversed following gabazine treatment, with larvae spending less than 20% of their time in the water stream containing the predator odour (Fig. 2b). The best fit log-linear model for both experiments was the saturated model containing the run × treatment × preference interaction. Removal of this interaction term in model selection caused a highly significant increase in deviance (predator odour versus seawater, likelihood ratio chi-square 11.82, d.f. = 3, $p = 0.008$; predator versus non-predator odour, likelihood ratio chi-square 10.24, d.f. = 3, $p = 0.017$), demonstrating that gabazine had a significant effect on the behavioural preference of high-CO_2-treated fish, but not control fish.

Damselfish larvae reared in high CO_2 exhibited a dramatic shift in behavioural lateralization, which was completely corrected by gabazine treatment (Fig. 3). A significant number of larvae from control conditions exhibited a higher preference for making either left or right turns than expected by chance (G-test: $G = 34.87$; d.f. = 5; $p < 0.0001$). Exposure to ~900 μatm CO_2 for four days totally disrupted this individual lateralization, making all individuals turn at random in a non-lateralized manner ($G = 7.05$; d.f. = 5; $p > 0.1$), as has been observed in previous experiments[5]. As a result, the absolute lateralization index of fish in the high-CO_2 group was significantly less than that of control fish (t-test; $t_{w(48)} = 3.04$; $p < 0.01$). Gabazine treatment reversed this effect, with the larvae regaining the lateralization level of the controls ($t_{(43)} = 0.57$; $p > 0.5$), Gabazine only had a significant effect on the high-CO_2 group (paired t-test: $t_{(14)} = 6.82$; $p < 0.001$), with lateralization remaining unaffected by gabazine treatment in the controls ($t_{(14)} = 1.21$; $p > 0.1$) (Fig. 3).

Our results clearly indicate that altered GABA-A receptor activity mediates the effects of high CO_2 on neural function in larval fishes, including loss of lateralization and reversal of olfactory responses. Gabazine is highly specific in its action as a GABA-A receptor antagonist[15], and we are not aware of any reports indicating other actions of this substance. We propose that changes in Cl^- and/or HCO_3^- gradients over neuronal membranes caused by acid–base regulation in CO_2-exposed fish causes a reversal of GABA-A receptor function (from inhibition to excitation) in some neuronal circuits. Normally, opening of the GABA-A receptor leads to an influx of Cl^- and HCO_3^- over the neuronal membrane, resulting in hyperpolarization of the neuron. If the gradients of these anions are altered so that there is a net outflux of anions on GABA-A receptor opening, the result is a depolarization of the neuron. Such changes in the ionic environment are expected from the way in which fish compensate for CO_2-induced acidosis: they accumulate HCO_3^- while releasing Cl^- and H^+ to the water[13,14]. The resultant fall in extracellular Cl^-, possibly combined with intracellular HCO_3^- accumulation, could then lead to Cl^- and HCO_3^- outflow from neurons and depolarization when GABA-A receptors are activated.

The ion channel of GABA-A receptors is about five times more permeable to Cl^- than to HCO_3^- (ref. 9) in mammals, so a fall in extracellular Cl^- would have a stronger effect on the direction of ion fluxes through the GABA-A receptor than a change in HCO_3^-. However, the selectivity of anion conductances of GABA-A receptors in fish has as far as we know not been studied, and it is possible that HCO_3^- plays a larger role in GABA-A receptor function in fish. The juvenile clownfish and damselfish studied here were much too small to enable blood sampling. However, larval fish are likely to have similar mechanisms for acid–base regulation to adult fish[14], and the early ontogenetic development of gills in fish is associated with the capacity for ion regulation during early life[16].

Reversal of GABA-A receptor function provides a mechanistic link between high CO_2 and brain function in animals. Altered GABA function due to a rise in CO_2 could have widespread and hitherto unrecognized effects on animals and ecosystems, because GABA-A receptors are phylogenetically old, occurring in both vertebrates and primitive invertebrates, and play a ubiquitous role as inhibitory components in neural circuits, and in the formation of such circuits[17,18]. Given the ubiquity of GABA-A receptors, there is good reason to expect that neuronal effects of high CO_2 levels are not confined to early life stages of coral reef fish. However, we predict that GABAergic effects of high CO_2 are more likely to occur in aquatic systems than in terrestrial systems, because water breathers generally have much lower plasma CO_2 and HCO_3^- levels than air breathers, usually differing by at least a factor of 10 (ref. 19). The reason for this is that CO_2 has a much higher solubility than O_2 in water, making water breathers lose most of their metabolic CO_2 while taking up O_2 over their respiratory surface. As a result, changes in ambient CO_2 levels have a much larger impact on the gradient of CO_2 over the respiratory surface in water breathers when compared with air breathers.

Even among water breathers, there will probably be differences in the sensitivity to GABAergic effects of increasing CO_2 levels. Some marine invertebrates do not fully compensate the tissue acidosis that occurs when exposed to high CO_2 (refs 20,21). Although this may make them susceptible to metabolic impacts of acidosis, the incomplete compensation of acid–base relevant ions is likely to make them less susceptible to effects on GABA-A receptor function. In contrast, some crustaceans and most teleost fish exhibit complete, or near-complete, compensation of acidosis by HCO_3^- accumulation and Cl^- efflux[13,14,20,21], and this could make them much more susceptible to effects of CO_2 on GABA-A receptor function. Ironically, it could be that aquatic animals with the best-developed acid–base regulation will also be most susceptible to disruption of GABA-A receptor function by rising CO_2.

Although larval fishes will probably experience relatively stable CO_2 conditions that are in equilibrium with the atmosphere during their pelagic stage in the open ocean[9], they may experience significant diurnal fluctuations in pCO_2 (partial pressure of CO_2) once settled to the reef, temporarily approaching the levels used in our high-CO_2 treatment[22,23]. However, previous research shows that behavioural impairment only occurs after several days' exposure to high CO_2 and impairment is retained for several days after larvae are returned to low-pCO_2 conditions. Therefore, short-term fluctuations do not seem to mediate the behavioural effects of high CO_2 after the larvae settle to the reef[2]. As the clownfish larvae were reared from hatching in high-CO_2 water, and still showed striking olfactory abnormalities, processes such as acclimation or phenotypic plasticity are apparently unable to reverse the effects of high CO_2, at least during this early stage of life. Individual variation in behavioural responses has been observed at intermediate CO_2 levels (~700 μatm; ref. 2), which could provide some opportunity for selection of CO_2-tolerant individuals. However, the heritability of this variation, and thus the potential for adaptation, is unknown.

Species and early life-stages of fish with very high rates of oxygen consumption, including the larvae of reef fishes[24] and highly active pelagic species, are likely to be among the most susceptible to changes in ambient CO_2 because their high rates of gas exchange can be expected to result in particularly low blood pCO_2 that approaches ambient pCO_2. For example, pCO_2 levels in blood fall sharply as gas exchange increases during fast swimming in mackerel, with arterial pCO_2 approaching 0.13 kPa (ref. 25). At such a low blood pCO_2, a future rise in water pCO_2 from 0.04 kPa (corresponding to today's ~400 μatm) to 0.09 kPa (corresponding to 900 μatm) would lead to significant changes in blood pCO_2. Thus, we suggest that the effects of rising ambient CO_2 on GABA-mediated behavioural changes are likely to occur first in active pelagic species and larval stages. This could include many species that are important to commercial and artisanal fisheries, and they should be a priority for further research.

Methods

Clownfish (*Amphiprion percula*) were reared at James Cook University from wild-caught breeding pairs using established techniques[3]. Larvae from two different parental pairs were used. Immediately after hatching the larvae from each clutch were divided into two equal-sized groups and transferred to 60 l aquaria with either control or high-CO_2 water. Larvae were reared under these conditions until the end of their pelagic larval stage (11 days), when they are competent to settle to benthic habitat. A two-channel choice flume[26] was used to test the behavioural responses of settlement-stage larvae to predator odour, both before (run 1) and after (run 2) gabazine treatment (30 min exposure to a 4 mg l^{-1} solution in seawater) or a seawater sham treatment. Two different odour combinations were used. (1) Larvae were given the choice of a water stream containing the odour of a common predator (rockcod, *Cephalopholis cyanostigma*) versus water containing no additional odour. (2) Larvae were given the choice of a water stream containing predatory rockcod odour versus water containing odour of a non-predator (herbivorous rabbitfish, *Siganus corallinus*).

Settlement-stage damselfish (yellowtail demoiselle *Neopomacentrus azysron*, ~18 days after hatching[27]) were caught in light traps[28] at Lizard Island, Great Barrier Reef, during December 2010. Equal-sized groups were transferred to 35 l aquaria with either control or high-CO_2 water (Supplementary Table S1). After four days treatment a detour test[5,29] was used to test the behavioural lateralization[30] of control and high-CO_2-exposed damselfish larvae, before (run 1) and after gabazine (run 2) treatment. In the detour test each damselfish was placed in a two-way T-maze corridor, where at the ends it had to turn either right or left. Each fish was tested in two runs, where the direction of 10 turns was recorded in each run. During run 2, half the fish had been treated with gabazine and the others were given a seawater sham treatment (as with clownfish).

The total lengths of the clownfish and damselfish used were approximately 7 mm and 12 mm, respectively.

See Supplementary Methods for full method details.

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Author contributions

G.E.N. and P.L.M. devised the study. G.E.N., P.L.M. and P.D. designed the experiments. G.E.N., P.D., D.L.D., P.L.M., M.I.M. and C.S. conducted the experiments. S-A.W. developed equipment and conducted the chemical analyses. P.L.M. and P.D. conducted the statistical analyses. All authors contributed to writing the paper.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper on www.nature.com/natureclimatechange. Reprints and permissions information is available online at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to G.E.N.